An ordered sequential mechanism for Factor IX and Factor IXa binding to platelet receptors in the assembly of the Factor X-activating complex

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To define the contributions of the Ω-loop of the Gla (γ-carboxyglutamic acid) domain and the EGF2 (second epidermal growth factor) domain of FIXa (Factor IXa) in the assembly of the FX-activating complex on activated platelets and phospholipid membranes, three recombinant FIXa chimeras were prepared with corresponding residues from the homologous coagulation protein, FVII: (i) Gly^4–Gln^{11} (FIXa7Ωloop), (ii) Cys^{88}–Cys^{124} (FIXa-7EGF2), and (iii) both Gly^4–Gln^{11} and Cys^{88}–Cys^{124} (FIXa7Ω-loop7EGF2). All three chimeras were similar to wild-type FIXa, as assessed by SDS/PAGE, active-site titration, content of Gla residues, activation rates by FXIa and rates of FXa generation in solution. Titrations of FX or FVIIIa on SFLLRN peptide-activated platelets and on phospholipid vesicles in the presence of FVIIIa revealed normal substrate and cofactor binding to all chimeras. In kinetic assays in the presence of phospholipid vesicles and FVIIIa, compared with wild-type FIXa $K_{d,\,app} \sim 4$ nM, the FIX7Ωloop chimera showed a 1.6-fold increase in $K_{d,\,app}$, the FIX7EGF2 chimera had a 7.4-fold increase in $K_{d,\,app}$, and the FIX7Ωloop7EGF2 chimera showed a 21-fold increase in $K_{d,\,app}$. In kinetic assays and equilibrium platelet-binding assays with activated platelets and FVIIIa, compared with wild-type FIXa ($V_{max} \sim 5$ nM min^{-1}; $K_{d,\,app} \sim 0.5$ nM; $B_{max} \sim 550$ sites/platelet; $K_d \sim 0.5$ nM), the FIX7Ωloop chimera displayed 2-fold decreases in V_{max} and B_{max} and 2-fold increases in $K_{d,\,app}$ and K_d. The FIX7EGF2 chimera displayed 2-fold decreases in V_{max} and B_{max} and 10-fold increases in $K_{d,\,app}$ and K_d. The FIX7Ωloop7EGF2 chimera showed non-saturable curves and severely impaired rates of FXa generation, and non-saturable, non-specific, low-level binding to activated platelets. Thus both the Gla domain Ω-loop (Gly^4–Gln^{11}) and the EGF2 domain (Cys^{88}–Cys^{124}) are required to mediate the normal assembly of the FX-activating complex on activated platelets and on phospholipid membranes.

Key words: Factor IXa, Factor X activation, phospholipid membrane, surface binding, surface catalysis, vitamin K-dependent coagulation enzyme.

INTRODUCTION

The assembly of the FX (Factor X)-activating complex on the surface of activated platelets is essential for the maintenance of normal haemostasis *in vivo*, as patients with deficiencies of the components of this complex, including FIX [1], FVIII [2], FX [3] and platelet-binding sites [4,5], experience serious life-threatening bleeding complications. This complex comprises an enzyme (FIXa), a cofactor (FVIIIa) and a substrate (FX), which are assembled on a productive complex on the surface of activated platelets or on anionic phospholipid membranes with a resultant $> 10^7$-fold increase in the catalytic efficiency (k_{cat}/K_m) of FX activation [6–15].

Both FIXa and its plasma-circulating zymogen FIX bind to platelets and to phospholipid vesicles containing PS (phosphatidylserine). Previous studies from our laboratory have demonstrated that several classes of specific high-affinity receptors are exposed on the surface membranes of platelets activated with low concentrations (< 1 nM) of thrombin, but not with ADP, even at very high (100 μM) concentrations [8–12]. Activation-specific binding sites have been identified for the following ligands: (i) FIX ($n \sim 250$ sites/platelet; $K_d \sim 2.5$ nM), sites that can also be occupied by FIXa [9,10]; (ii) FIXa ($n \sim 250$ sites/platelet; $K_d \sim 2.5$ nM), sites that are specific for the enzyme, the affinity of which is enhanced ($K_d \sim 0.5$ nM) in the presence of both FVIIIa and FX [9,10]; (iii) FVIII ($n \sim 450$ sites/platelet; $K_d \sim 3.0$ nM) [13,14,16–19]; (iv) FVIIIa ($n \sim 1200$ sites/platelet; $K_d \sim 0.8$ nM) [14,16]; (v) FX and prothrombin ($n \sim 15{,}000$ sites/platelet; $K_d \sim 300$ nM), a high-capacity, low-affinity shared site [11,12]; and (vi) FX ($n \sim 1000$ sites/platelet; $K_d \sim 5$ nM), a specific FX-binding site comprising bound FVIIIa [12,14,20]. Occupancy of these binding sites on activated, but not unactivated, platelets is closely correlated with enhanced rates of FX activation so that the catalytic efficiency (k_{cat}/K_m) is increased $> 2 \times 10^7$-fold when all these binding sites are occupied [8,10,14,20], demonstrating that receptor occupancy is physiologically relevant. Therefore it is a matter of great interest and importance to define the molecular domains within the zymogen, the enzyme, the cofactor and the substrate that mediate these interactions, and the sequential mechanisms and requirements for the assembly of this important enzyme–cofactor–substrate complex.

In an attempt to identify the molecular domains within FIX and FIXa that mediate their interactions with their shared and specific binding sites, we have previously utilized chimeric FIX/FIXa proteins, site-directed mutant proteins and conformationally constrained synthetic peptides. These experiments suggest that both FIX and FIXa utilize the Ω-loop (Gly^4–Gln^{11}) of the Gla (γ-carboxyglutamic acid) domain to bind to an average of ~ 250 shared binding sites/platelet, whereas FIXa, but not FIX, utilizes residues Cys^{88}–Cys^{109} of the EGF2 (second epidermal

Abbreviations used: DEGR, dansyl-Glu-Gly-Arg-chloromethylketone; EGF2 domain, second epidermal growth factor domain; FRT, Flp recombination target; (r)FX, FXa etc., (recombinant) Factor X, Factor Xa etc; $FIX_{N/wt}$, normal plasma-derived/wild-type FIX; Gla, γ-carboxyglutamic acid; HT, Hepes-Tyrodes; PC, phosphatidylcholine; PS, phosphatidylserine; TBS, Tris-buffered saline; V_{max}^{VIIIa}, maximum velocity of FXa generation at a saturating concentration of FVIIIa; V_{max}^{IXa}, maximum velocity of FXa generation at a saturating concentration of FIXa; wt, wild-type.

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growth factor) domain to bind to an additional ~250 specific binding sites/platelet [21–29]. However, the relative functional contributions of these interactions to the assembly of the FX-activating complex are unknown, as is the sequential mechanism required for FIX and FX activation on the platelet surface. The present studies with FIX chimeric proteins suggest a novel mechanism that explains the relative contributions of the Gla domain and the EGF2 domain of FIX/FIXa to FIX activation and FX activation, and resolve previously confusing issues concerning the functional relationship between the shared FIX/FIXa-binding site and the specific FIXa-binding site on activated platelets and on phospholipid membranes.

EXPERIMENTAL

Materials

The Flp-In™ expression vector pcDNA5/FRT/V5-His-TOPO® (where FRT is the Flp recombination target, see below) was purchased from Invitrogen; cDNA polymerase from *Pyrococcus furiosis* (*Pfu* DNA polymerase), Taq DNA polymerase and reaction buffer from Stratagene; oligonucleotides from Gibco BRL; materials for plasmid purification, PCR product purification and transfection of mammalian cells from Promega or from Qiagen; Flp-In™ HEK-293 cells were from Invitrogen; Dulbecco's modification of Eagle's medium from Mediatech (Herndon, VA, U.S.A.); vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) from Abbott Laboratories (Chicago, IL, U.S.A.); antibodies for FIX ELISA from Enzyme Research Laboratories (South Bend, IN, U.S.A.); Q-Sepharose anion-exchange resin from Sigma, and Centricon Plus-20 concentration units from Millipore. Hepes, Hepps, Tris, fatty acid-free BSA, heparin from porcine intestinal mucosa, benzamidine and other reagents were purchased from Sigma. Human FIX, human FX and human anti-thrombin III were obtained from Enzyme Research Laboratories, and human FVII, human FVIIa, human FIX and human FIXa with the active site inhibited with dansyl-Glu-Gly-Arg-chloromethylketone (FIXa–DEGR) from Haematologic Technologies Inc. (Essex Junction, VT, U.S.A.). High-purity recombinant human FVIII was generously given by Baxter Healthcare Corp. (Duarte, CA, U.S.A.). Thrombin was purchased from Sigma, the chromogenic substrate S-2765 (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-*p*-nitroanilide) from Dia Pharma Group (Stockholm, Sweden), and bovine brain PS and PC (phosphatidylcholine, L-α-dioleoylphosphatidylcholine) from Avanti Polar Lipids (Birmingham, AL, U.S.A.).

Buffers

TBS (Tris-buffered saline) containing 10 mM Tris and 150 mM NaCl, pH 7.4, was supplemented with 2 mM benzamidine, 2 mM EDTA, 5 mM $CaCl_2$ where indicated. HT (Hepes-Tyrodes) buffer for FXa generation assays contained 15 mM Hepes, 126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 375 μM NaH_2PO_4, 5.6 mM glucose, pH 7.4, supplemented with BSA (0.5 mg/ml) and 5 mM $CaCl_2$.

Platelet-surface FXa generation by FVIIa

FVIIa at indicated concentrations up to 50 nM was added to reaction vessels containing FVIIIa (5 units/ml) and SFLLRN peptide (5 μM)-activated platelets (5×10^7/ml) in HT buffer supplemented with BSA (0.5 mg/ml) and $CaCl_2$ (5 mM). FXa generation was initiated by addition of 250 nM FX, was allowed to proceed for 2 min at 37 °C and was stopped by addition of 10 mM EDTA. The amount of FXa generated was determined by using its chromogenic substrate S2765 as described in [29]. Titrations of FIXa proteins at concentrations up to 5 nM were included in the platelet-surface FXa generation assay as controls.

Competition studies with FIXa in platelet-mediated FX-activating complex

Competitors (DEGR–FIXa, FVIIa, FVII), at the indicated concentrations, were pre-incubated with normal plasma-derived FIXa (0.1 nM), SFLLRN (5 μM)-activated platelets (5×10^7/ml) and FVIIIa (5 units/ml) at 37 °C for 15 min before addition of 250 nM FX to start the reaction, and FX activation by residual FIXa was allowed to proceed for 2 min and was stopped by the addition of EDTA (10 mM). The amount of FXa generated was determined by using its chromogenic substrate S2765 as described in [29].

Site-directed mutagenesis

The cDNA constructs pCMV5/fIXwt and pCMV5/fIX7EGF2, expressing the wild-type recombinant FIX and the recombinant FIX chimera rfIX7EGF2 respectively [29], served as templates for mutagenesis. A modified QuikChange™ method [30], which has the advantage of generating more than 10 bp of mutations in one PCR, was used to generate the new constructs pCMV5/fIX7Ωloop and pCMV5/fIX7Ωloop7EGF. Mutagenesis primers were designed so that the mismatch regions encoding the desired 5-amino-acid mutations in the Gly^4–Gln^{11} region of FIX were flanked by 25-bp complementary sequences at both ends. Specifically, a mutagenesis PCR mixture (50 μl) contained 100 ng of DNA template (pCMV5/fIXwt or pCMV/fIX7EGF2), 200 ng of each primer (forward, 5′-CGGCCAAAGAGGTATAATTCAgcgttcTTGGAAGAGctgcggccgGGGAACCTTGAGAGAGAATGTATGG-3′; reverse, 5′-CCATACATTCTCTCTCAAGGTTCCCcggccgcagCTCTTCCAAgaacgcTGAATTATACCTCTTTGGCCG-3′; the lower case letters designate the mutated sequences), 10 mM dNTP mix and 2.5 units of PfuTurbo™ (Stratagene). The PCR cycle comprised a denaturing step at 95 °C for 30 s, followed by 18 cycles of 30 s at 95 °C, and 18 min at 68 °C. The parental DNA template was digested by Dpn I for 3 h at 37 °C, and the product was then transformed into Epicurian Coli® supercompetent cells (Stratagene). Single colonies were picked from the transformation plates, cultured and the mutation-containing plasmid DNA was isolated from transformed cells using the Wizard™ Plasmid miniprep kit (Promega). The size of plasmid DNA was confirmed by agarose gel electrophoresis and DNA sequencing was performed by ACGT Inc. (Northbrook, IL, U.S.A.).

Generation of the Flp-In™ expression plasmid

The Flp-In™ expression system, which generates stable mammalian expression cell lines via Flp recombinase-mediated integration, was chosen to express the designed FIX chimeras. The cDNA constructs pCMV5/fIX7Ωloop and pCMV5/fIX7Ωloop7EGF were subcloned into the Flp-In™ expression vector pcDNA5/FRT by TA cloning as instructed by the manufacturer (Invitrogen).

Transfection of Flp-In™-293 cells

Flp-In™-293 cells (Invitrogen, Carlsbad, CA) are a modified HEK-293 cell line with the FRT site. Transfection was carried out using the calcium phosphate precipitation technique, using the Profection® Mammalian Transfection Systems kit (Promega). Each 100-mm diameter culture dish received 1 μg of the appropriate FIX expression vector (pcDNA5/FRT/fIX7Ωloop or pcDNA5/FRT/fIX7Ωloop7EGF2) and 9 μg of the plasmid

pOG44 (Invitrogen), which transiently expresses the recombinase mediating the integration. DNA-integrated cells were selected using 100 μg/ml hygromycin.

Purification of FIX proteins

Cell lines with the highest protein expression levels (determined by ELISA) were selected for recombinant FIX purification. Confluent cells were washed with PBS twice and then switched to serum-free medium supplemented with vitamin K for 24 h. Fresh serum-free medium was added and conditioned medium was collected every 72 h. The conditioned medium was supplemented with 5 mM benzamidine and 5 mM EDTA and filtered through a cellulose acetate filter (0.45 μm pore size) to remove cell debris. The medium was stored at 4 °C until the day of purification. Purification of recombinant FIX using ion-exchange chromatography on Q-Sepharose was carried out as described in [31]. For 500 ml of conditioned serum-free medium, a 1 ml slurry of Q-Sepharose beads was used. The Q-Sepharose beads were first thoroughly equilibrated in the equilibration buffer (TBS with or without 5 mM benzamidine and/or 5 mM EDTA), and then packed in the chromatography column. The conditioned serum-free medium was then subjected to chromatography. The column was washed with 30 column vol. (~60 ml) of equilibration buffer and then further washed with 20 column vol. (~40 ml) of TBS supplemented with 2 mM benzamidine to remove the EDTA. Recombinant FIX proteins were then eluted with TBS containing 2 mM benzamidine and 5 mM $CaCl_2$. Fractions were concentrated to ~3 ml using Centricon Plus-20 (10 000 molecular mass cut-off, Millipore) and dialysed in TBS buffer.

Protein concentration

Protein concentrations were initially determined using the bicinchoninic acid assay [32], and were then corrected utilizing the results of active-site titration as described below.

Gla analysis

Analysis of FIX proteins for Gla was kindly carried out by Dr Rodney Camire (Children's Hospital of Philadelphia, Philadelphia, PA, U.S.A.), as described in [29].

Activation of FIX proteins by FXIa

FIX proteins were converted into their active enzymatic forms as follows. FIX proteins (1 μM) were diluted in HT buffer supplemented with 5 mM $CaCl_2$. FXIa (5 nM) was added at a 1/200 molar ratio and the reactions were incubated at 37 °C for 90 min. Complete activation was confirmed by SDS/PAGE and silver staining, and by active-site titration with anti-thrombin III.

Active-site titration of FIXa proteins

In active-site titration, 10 μl of FIXa protein (100 nM) was incubated with 10 μl of anti-thrombin III dilutions (0–100 nM) in HT buffer (15 mM Hepes, 126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 375 μM NaH_2PO_4 and 5.6 mM glucose, pH 7.2) supplemented with BSA (1 mg/ml), heparin (20 μg/ml) and $CaCl_2$ (5 mM) at 37 °C for 15 min, and the reaction was diluted by adding HT buffer to 200 μl. Residual FIXa activity was examined by assaying FXa generation activity in the presence of FVIIIa (5 units/ml), FX (400 nM) and PS/PC vesicles (20 μM). FXa generation reaction was allowed to proceed at 37 °C for 2 min and stopped by the addition of 50 μl of stopping buffer (50 mM Hepes, 175 mM NaCl and 20 mM EDTA, pH 8.1). The amount of FXa generated was determined by using the chromogenic substrate S2765 as described in [29].

Solution-phase FXa generation

For solution-phase FX titrations, FIXa proteins were added to 15 nM in reaction vessels containing HT buffer supplemented with BSA (2 mg/ml) and 5 mM $CaCl_2$, and the reaction was initiated by the addition of FX at the indicated concentration. For solution-phase FIXa titrations, FIXa was titrated to the indicated concentration in reaction vessels containing HT buffer supplemented with BSA (2 mg/ml) and 5 mM $CaCl_2$, and the reaction was initiated by the addition of 2 μM FX. Reactions were allowed to proceed for 1 h at 37 °C before the addition of 10 mM EDTA to stop the reaction. The amount of FXa generated was determined using the chromogenic substrate S2765 as described in [29].

FXa generation assays on activated platelets or PC/PS (3:1) vesicles

For platelet studies the FX activation vessel contained 1 nM FIXa, 5 units/ml FVIIIa and SFLLRN-activated platelets (5×10^7 platelets/ml) in HT buffer supplemented with BSA (2 mg/ml) and 5 mM $CaCl_2$. Extruded phospholipid vesicles comprising 75 % PC and 25 % PS (mol/mol) were prepared as described in [29]. FXa generation assays were performed in the presence of activated platelets or PC/PS vesicles at appropriate concentrations, following previously described methods [29]. The reaction was initiated by the addition of FX to the indicated concentration and allowed to proceed for 2 min at 37 °C. Then 10 mM EDTA was added to stop the reaction and the FXa generation was measured as indicated above.

Determination of $K_{d,\,app(FVIIIa)}$ and FX activation velocity in the presence of saturating FVIIIa (V_{max}^{VIIIa})

FVIIIa at indicated concentrations was titrated into the FX-activation vessel containing 0.5 nM FIXa and either SFLLRN-activated platelets (5×10^7 platelets/ml) or PS/PC vesicles. The reaction was initiated by the addition of 250 nM FX and allowed to proceed for 2 min at 37 °C. Then 10 mM EDTA was added to stop the reaction. The amount of FXa generated was determined as above. V_{max}^{VIIIa} was defined as the maximum velocity of FXa generation at a saturating concentration of FVIIIa under the experimental conditions described. $K_{d,\,app(FVIIIa)}$ was defined as the concentration of FVIIIa required to achieve half-maximal rates of FX activation.

Determination of $K_{d,\,app(FIXa)}$ and FX activation velocity in the presence of saturating FIXa (V_{max}^{IXa})

FIXa at indicated concentrations was titrated into the FX activation vessel containing 5 units/ml FVIIIa and SFLLRN-activated platelets or PS/PC vesicles. The reaction was initiated by the addition of 250 nM FX and allowed to proceed for 2 min at 37 °C. Then 10 mM EDTA was added to stop the reaction. The amount of FXa generated was determined as above. V_{max}^{IXa} was defined as the maximum velocity of FXa generation at a saturating concentration of FIXa under the experimental conditions described. $K_{d,\,(app)FIXa}$ was equivalent to the EC_{50} for FIXa concentration, i.e. the concentration of FIXa required to achieve half-maximal rates of FXa generation under the experimental conditions described.

Data analysis

FXa generation rates from all reactions described here were fitted to a hyperbolic curve using a non-linear least-squares fit, as described in [29]. Kinetic constants, including K_m, V_{max}, $K_{d,\,app(FVIIIa)}$, V_{max}^{VIIIa}, $K_{d,\,app(FIXa)}$ and V_{max}^{IXa}, were derived using

KaleidaGraph Software as described in [29]. Values for catalytic-centre activity ('turnover number', k_{cat}) were calculated as previously described [26] by dividing values of V_{max} by the number of bound enzyme (FIXa) molecules, determined from equilibrium-binding measurements. Statistical analysis was carried out using ANOVA followed by pair-wise comparisons with the Bonferroni adjustment procedure for multiple comparison, maintaining an experiment-wise type 1 error level of 0.05 [33], as described in [29].

Equilibrium binding of FIXa to activated platelets in the presence of FVIIIa and FX

Native FIX and recombinant FIX mutants were radiolabelled with ^{125}I using the Iodogen method, and converted into active FIXa using FXIa [9]. FIXa proteins (50 μl), at the indicated concentrations, were incubated with 50 μl of SFLLRN-activated platelets (3×10^8/ml) in the presence of FVIIIa (5 units/ml) and FX (2.5 μM) at 37 °C for 20 min, and the incubation mixture was transferred into a microcentrifuge tube and centrifuged through a mixture of silicone oil, as described in [9]. Bound FIXa was separated from unbound FIXa by removing the tips of the microcentrifuge tube, and the radioactivity of the bound and unbound fractions were assayed using a gamma counter (Perkin-Elmer). Non-specific FIXa binding, examined in the presence of a 100-fold excess of unlabelled FIXa protein, was subtracted from the total observed FIXa binding to determine specific FIXa binding.

RESULTS

Comparisons of FIXa, FVII and FVIIa in platelet-mediated FX-activating complex assembly

FVII was chosen as the mutation template for making FIXa 'loss-of-function' chimeras. It is known that recombinant FVIIa activates FX *in vivo* at concentrations well above the FVII concentration in plasma [34,35]. Control experiments were carried out to show that, under identical experimental conditions with SFLLRN-activated platelets (5×10^7/ml) and the cofactor FVIIIa (5 units/ml) present, FVIIa was extremely ineffective in generating FXa (Figure 1A). The maximal velocity observed was $\sim$0.1 nM FXa/min at 50 nM FVIIa, and $K_{d,\,app}$ could not be determined, since rates of FXa generation in FVIIa titrations were non-saturable in contrast to titrations with native FIXa (Figure 1A, inset; $V_{max} \sim 6$ nM FXa/min, $K_{d,\,app} \sim 0.5$ nM). A second control experiment (Figure 1B) was performed in which DEGR–FIXa, FVII and FVIIa were titrated as competitors to displace native FIXa (0.1 nM) from the assembled FX-activating complex comprising activated platelets (5×10^7/ml) and FVIIIa (5 units/ml). Native FIXa was completely displaced by DEGR–FIXa ($K_i \sim 0.5$ nM), whereas FVIIa displaced only 25 % native FIXa at a concentration of 50 nM, and FVII had no effect. In agreement with previous results that FVIIa binds to activated platelets with low affinity ($K_d \sim 150$ nM) [36] compared with FIXa ($K_d \sim$ 0.5 nM/2.5 nM in the presence/absence of FVIIIa and FX), the results of these experiments indicate that residues in the FVII/FVIIa molecule are relatively ineffective in platelet-mediated FX-activating complex assembly and therefore we are justified in using mutation templates for chimeric FIX proteins.

Characterization of chimeric proteins

The chimeric FIX proteins, which contained only the full-length sequence of the mature proteins without a His tag, were expressed

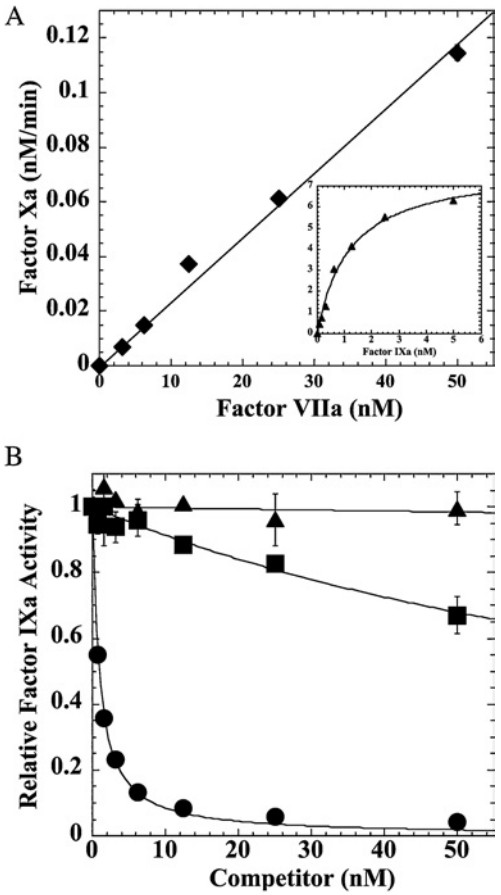


Figure 1 Platelet-surface FXa generation by FVIIa and displacement of FIXa from platelet-mediated FX-activating complex

(**A**) FX was activated by FVIIa (up to 50 nM) or FIXa (up to 5 nM) on the platelet surface, as described in the Experimental section. Rates of FXa generation were determined and plotted as a function of input FVIIa or FIXa (inset) concentrations. ◆, FVIIa; ▲, FIXa. (**B**) FIXa activities (relative) in activating FX on the platelet surface were determined in the presence of increasing concentrations of competitors. ●, DEGR–FIXa; ■, FVIIa; ▲, FVII. The results are expressed as the means ± S.E.M. for three determinations.

in Flp-In™-293 cells and purified from conditioned serum-free medium. The amino acids of FIX within the Ω-loop of the Gla domain (Gly^4-Lys-Leu-Gla-Gla-Phe-Val-Gln^{11}) were exchanged with those of FVII (Ala^4-Phe-Leu-Gla-Gla-Leu-Arg-Pro^{11}) to produce the chimeric proteins utilized in the present study. The primary sequences of the EGF2 domain of FIX that were changed to homologous residues in FVII have been published previously [29]. Each of the recombinant FIX proteins was found to co-migrate with normal plasma-derived FIX (FIX_N), suggesting normal translation and post-translational modification (results not shown). Additionally, each of the recombinant FIX proteins was found to have the expected number of Gla residues (12 mol of Gla/mol of protein): FIX_N, 13.0 ± 0.1; $rFIX_{wt}$, 10.9 ± 0.1; FIX7Ω-loop, 12.8 ± 0.01; rFIX7EGF2, 11.9 ± 0.01; rFIX7Ωloop7EGF2, 10.9 ± 0.1.

Each of the FIX proteins was activated with FXIa in solution. Both silver-stained gels and Western blotting of SDS/PAGE showed disappearance of the zymogen band ($\sim$70 kDa) and appearance of heavy ($\sim$28 kDa) and light chains ($\sim$18 kDa) of FIXa for all the recombinant FIXa proteins (results not shown). Complete activation of all FIX proteins was also supported by active-site titration with anti-thrombin III. The concentrations of

chimeric and normal proteins used in the active-site titrations were ~100 nM, as determined at A_{280} and with the bicinchoninic acid assay. The active site concentrations exhibited for the recombinant FIXa proteins were: FIX_N, 100 nM; $rFIX_{wt}$, 102 nM; FIX7Ωloop, 85 nM; rFIX7EGF2, 95 nM; rFIX7Ωloop7EGF2, 105 nM. For all subsequent experiments, the concentrations of chimeric, wild-type and plasma-derived FIXa molecules were adjusted according to the active site determinations.

To determine whether the mutations made to the recombinant FIX proteins within the putative surface-interacting domains affect their enzymatic activities, the FIXa activity of the chimeras was examined in solution. In the absence of surface, FX activation by FIXa is extremely inefficient ($K_m \sim 300\ \mu M$, $k_{cat} \sim 0.002\ s^{-1}$). When FX was titrated at concentrations up to 12 μM in solution, identical low FXa generation rates were observed for all the FIXa proteins (results not shown). Additionally, no significant difference in enzymatic activity of FIXa proteins was observed when the enzymes were titrated in solution (results not shown). This indicates that mutations in the proposed surface-interacting regions of FIXa do not alter its activity when no surface is provided.

FX titrations

Rates of FX activation by the FIXa chimeras were examined at variable FX concentrations in the presence of activated platelets (Figures 2A and 2B) or on extruded phospholipids containing negatively charged PS (Figure 2C). Providing a surface to the FXa generation assay differentiated the chimeric FIXa proteins from native FIXa, since in FX titrations, FIXa chimeras catalysed FX activation much less efficiently, as indicated by reduced V_{max} values (Table 1). However, no increase in $K_{m,\,app}$ values was observed for any of the chimeras, which suggests that the deficiency in FXa generation activity does not result from defective FX association with the enzymatic complex (Table 1). Whereas the observed K_m values in the substrate (FX) titration experiments are indicative of the affinity of substrate incorporation, K_m is not the true substrate dissociation constant (K_d or K_s). Rather, the reaction rate contributes to K_m ($K_m = K_d + k_{cat}/K_{on}$), which may explain why, when the reaction velocity is decreased for the FIXa chimeras, the $K_{m,app}$ values were significantly decreased (Table 1). However, because decreased $K_{m,app}$ values suggest even tighter substrate association with the enzyme complex, we concluded that the substrate incorporation was not interrupted for any of the chimeric proteins.

FVIIIa titrations

Enhancement of FIXa-catalysed FX activation by activated platelets or phospholipids depends on the effective incorporation of cofactor FVIIIa into the FX-activating complex. In order to assess FVIIIa interactions with FIXa chimeras, FVIIIa was titrated into the reaction mixture in the presence of activated platelets (Figure 3) or phospholipid vesicles (Figure 4). The maximal velocity of FX activation at saturating FVIIIa concentration (V_{max}^{VIIIa}, indicating the maximal extent of activity stimulation by the cofactor) was significantly decreased for all the FIXa chimeras compared with native FIXa. However, as shown in Table 2, the effective concentration at half maximal velocity [$K_{d,\,app(FVIIIa)}$] remained the same for all the FIXa proteins, indicating normal incorporation of the cofactor into the enzymatic complex in the presence of activated platelets or phospholipid vesicles. The sole exception was a significantly decreased value of $K_{d,\,app(FVIIIa)}$ for the double chimera, suggesting either that the affinity of FVIIIa binding in the presence of the double chimera is increased or that the decreased value of $K_{d,\,app(FVIIIa)}$ represents an artifact of extremely low rates of FXa generation characteristic of the double chimera (Figure 3C). In either case, we conclude that the defective rates of FXa generation observed with these chimeric proteins are not a consequence of defective interaction with the cofactor FVIIIa.

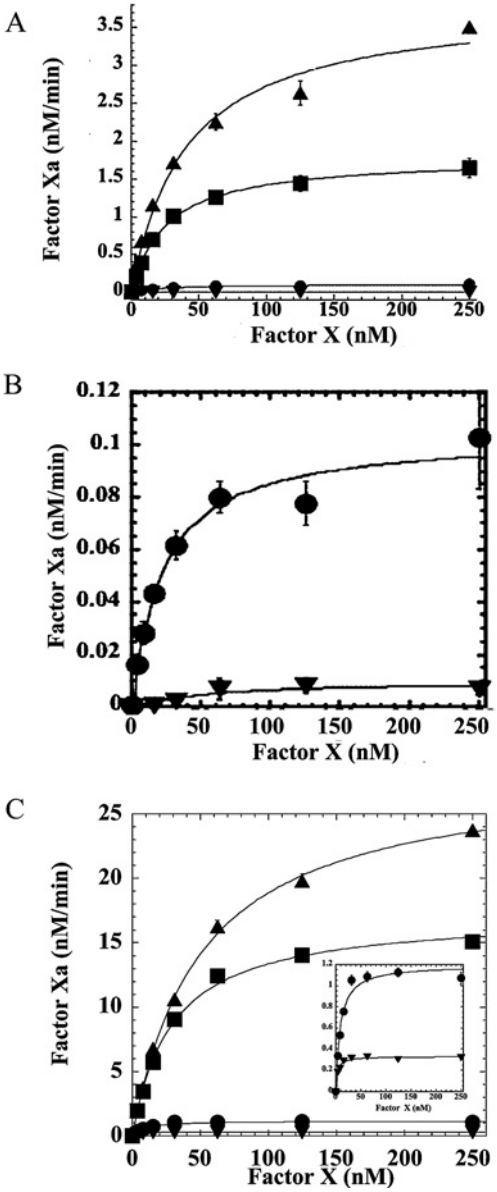


Figure 2 Determination of $K_{m,\,app}$ and V_{max} on SFLLRN-activated platelets or phospholipid vesicles (PC/PS, 3:1)

(**A**) FIXa proteins were diluted to 1 nM in HT buffer containing SFLLRN (5 μM)-activated platelets (5×10^7/ml). FVIIIa was added to 5 units/ml and FX was added to the indicated concentrations. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. (**B**) Results for rFIXa7EGF2 and rFIXa7Ωloop7EGF2 shown with rescaled ordinates. (**C**) FIXa proteins were diluted to 10 nM in HT buffer containing 20 μM PS/PC vesicles. FVIIIa was added to 5 units/ml and the reactions were initiated by addition of FX to the indicated concentrations. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. Inset, rescaled regression curves for rFIXa7EGF2 and rFIXa7Ωloop7EGF2. ▲, $FIXa_N$; ■, rFIXa7Ωloop; ●, rFIXa7EGF2; ▼, rFIXa7Ωloop7EGF2. The results are expressed as the means ± S.E.M. for three determinations.

FIXa titrations

Since the deficiency in FX-activating complex assembly observed with the chimeric proteins was not due to deficiencies in

Table 1 Kinetic parameters ($K_{m,\,app}$ and V_{max}) for FX activation by FIXa chimeras on SFLLRN-activated platelets and phospholipid vesicles (PC/PS, 3:1)

The results are expressed as the means ± S.E.M. of triplicate measurements for three independent experiments. *$P < 0.01$, †$P < 0.0001$ and ‡P = not significant when compared with $FIXa_N$. ND, not determined.

	Platelets			Phospholipids	
FIXa	$K_{m,\,app}$ (nM)	V_{max} (nM FXa · min^{-1})	k_{cat} (sec^{-1})	$K_{m,\,app}$ (nM)	V_{max} (nM FXa · min^{-1})
$FIXa_N$	40.5 ± 6.1	3.8 ± 0.1	3.2	50.2 ± 2.3	28.3 ± 0.5
FIX7Ωloop	25.9 ± 1.5*	1.8 ± 0.04*	3.8	28.6 ± 2.1*	17.2 ± 0.4*
FIX7EGF2	21.6 ± 3.9*	0.1 ± 0.006†	3.3	8.64 ± 1.5†	1.19 ± 0.5†
FIX7Ωloop7EGF2	50.8 ± 10.1‡	0.01 ± 0.001†	ND	3.11 ± 0.5†	0.33 ± 0.008†

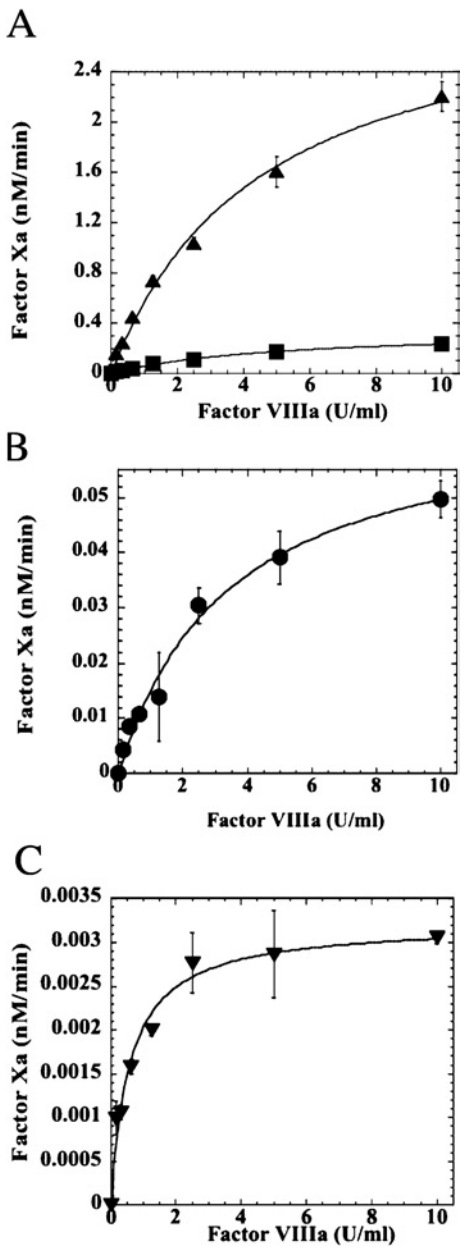


Figure 3 Determination of $K_{d,app(FVIIIa)}$ and V_{max}^{VIIIa} for FVIIIa stimulation of FXa generation on SFLLRN-activated platelets

FIXa proteins were diluted to 0.5 nM in HT buffer containing SFLLRN-activated platelets (5×10^7/ml). FVIIIa activated by thrombin (0.1 units/ml) was added to the indicated final concentration. Reactions were initiated by addition of FX to 250 nM. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. ▲, $FIXa_N$; ■, rFIXa7Ωloop; ●, rFIXa7EGF2; ▼, rFIXa7Ωloop7EGF2. Results for rFIXa7EGF2 (**B**) and rFIXa7Ωloop7EGF2 (**C**) are shown using rescaled ordinates. The results are expressed as the means ± S.E.M. for three determinations. U/ml, units/ml.

substrate or cofactor incorporation, enzyme titrations were carried out in the presence of activated platelets (Figure 5) or phospholipid vesicles (Figure 6). The values of maximal velocity at saturating concentrations of the enzyme (V_{max}^{IXa}) were significantly reduced for the FIXa chimeras compared with native FIXa, and the $K_{d,\,app(FIXa)}$ values were significantly increased for the chimeric proteins (Table 3). Specifically, on activated platelets the $K_{d,\,app(FIXa)}$ values for rIXa7Ωloop were ~2-fold increased, whereas rIXa7EGF2 displayed a ~10-fold increase in $K_{d,\,app(FIXa)}$. On phospholipids the $K_{d,\,app(FIXa)}$ value for rIXa7Ωloop was ~2-fold increased, whereas rIXa7EGF2 displayed a ~7-fold increase in $K_{d,\,app(FIXa)}$. The rIXa7Ωloop7EGF2 chimera displayed a >20-fold increase in $K_{d,\,app(FIXa)}$ on phospholipid vesicles, whereas the rIXa7Ωloop7EGF2 chimera displayed a completely non-saturable curve on activated platelets. The results indicated that enzyme incorporation was defective for all the FIXa chimeras, resulting in the decreased values of V_{max} for FX activation observed in the kinetic assays.

Equilibrium binding experiments

Equilibrium binding experiments with activated platelets were carried out to confirm the conclusion that the kinetic defects observed with the FIXa chimeric proteins arose from defective platelet binding. All the recombinant FIX wild-type and chimeric proteins, as well as purified plasma FIX, were radiolabelled with I^{125} (specific activity ~ 1×10^6 c.p.m./μg of protein) and activated to FIXa by FXIa in solution, as detected by SDS/PAGE and active-site titration. Both unlabelled and labelled wild-type and chimeric FIXa proteins were examined in FXa generation assays in which no differences in FIXa activity were observed, indicating that the FIXa proteins were not structurally or functionally impaired by the incorporation of radiolabel. The results of binding studies are shown in Figure 7 and the calculated values for affinity and stoichiometry are shown in Table 4. In the presence of FVIIIa (5 units/ml) and FX (2.5 μM), compared with native FIXa (K_d ~ 0.5 nM, B_{max} ~ 550 sites/platelet), the FIX7Ωloop chimera showed a 2-fold decrease in B_{max} and a 2-fold increase in $K_{d,\,app}$, the FIX7EGF2 chimera showed a 2-fold decrease in B_{max} and a 10-fold increase in $K_{d,\,app}$, and the FIX7Ωloop7EGF2 chimera displayed completely non-saturable, non-specific and low-level binding to activated platelets.

DISCUSSION

The present studies clarify the relationship between the shared FIX/FIXa platelet binding site, mediated by the Ω-loop

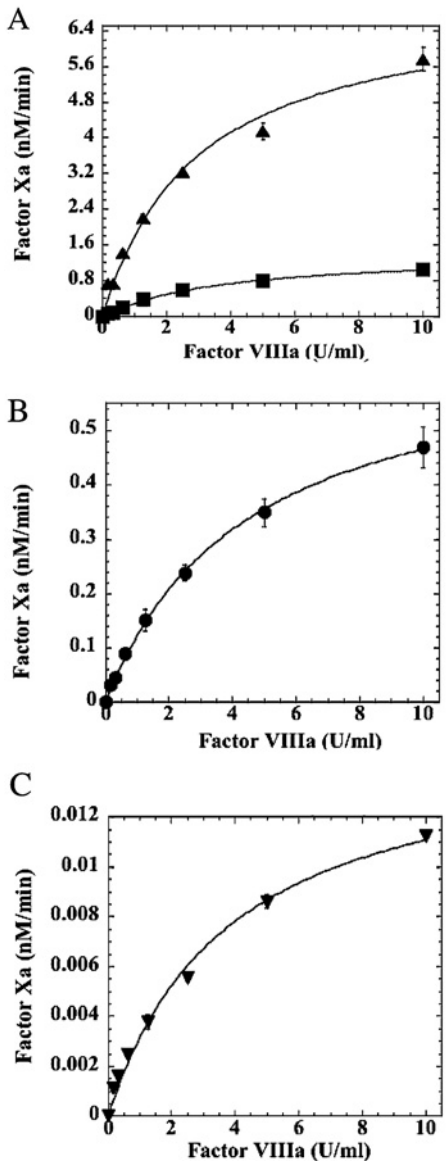


Figure 4 Determination of $K_{d,app(FVIIIa)}$ and V_{max}^{VIIIa} for FVIIIa stimulation of FXa generation on phospholipid vesicles (PC/PS, 3:1)

FIXa proteins were diluted to 0.5 nM in HT buffer containing 500 nM PS/PC vesicles. FVIII was added to the indicated final concentration and activated to FVIIIa by thrombin (0.1 units/ml). Reactions were initiated by addition of FX up to 250 nM. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. ▲, IXa_N; ■, rFIXa7Ωloop; ●, rFIXa7EGF2; ▼, rFIXa7Ωloop7EGF2. Regression curves for rFIXa7EGF2 (**B**) and rFIXa7Ωloop7EGF2 (**C**) are shown separately with rescaled ordinates. The results are expressed as the means ± S.E.M. for three determinations.

(Gly^4–Gln^{11}) of the Gla domain and the specific FIXa-binding site mediated by residues Cys^{88}–Cys^{109} of the EGF2 domain. Moreover, they provide the basis for proposing a model of the sequence of biochemical events leading to the assembly on the activated platelet surface of the FX-activating complex (Scheme 1). First and foremost, the present results demonstrate that both the Ω-loop of the Gla domain and residues Cys^{88}–Cys^{109} of the EGF2 domain are required for the binding of FIXa to its functional receptors and for efficient FX activation on the platelet surface. Whereas residues Gly^4–Gln^{11} of the Gla domain mediate the totality of FIX binding and half the FIXa binding (to the shared site), residues Cys^{88}–Cys^{109} of the EGF2 domain mediate FIXa

Table 2 FVIIIa titrations and kinetic parameters ($K_{d,app(FVIIIa)}$ and V_{max}^{VIIIa}) for stimulation of FXa generation on SFLLRN-activated platelets or phospholipid vesicles (PC/PS, 3:1)

The results are expressed as the means ± S.E.M. of triplicate measurements for three independent experiments. *$P < 0.0001$ and †P = not significant when compared with $FIXa_N$.

	Platelets		Phospholipids	
FIXa	$K_{d,app(FVIIIa)}$ (units · ml^{-1})	V_{max}^{VIIIa} (nM FXa · min^{-1})	$K_{d,app(FVIIIa)}$ (units · ml^{-1})	V_{max}^{VIIIa} (nM FXa · min^{-1})
$FIXa_N$	4.5 ± 0.6	3.1 ± 0.2	2.9 ± 0.5	7.1 ± 0.5
FIX7Ωloop	4.9 ± 0.6†	0.4 ± 0.02*	3.7 ± 0.3†	1.4 ± 0.05*
FIX7EGF2	3.4 ± 0.3†	0.06 ± 0.05*	4.4 ± 0.3†	0.67 ± 0.02*
FIX7Ωloop7EGF2	0.5 ± 0.3*	0.002 ± 0.001*	3.8 ± 0.6†	0.02 ± 0.001*

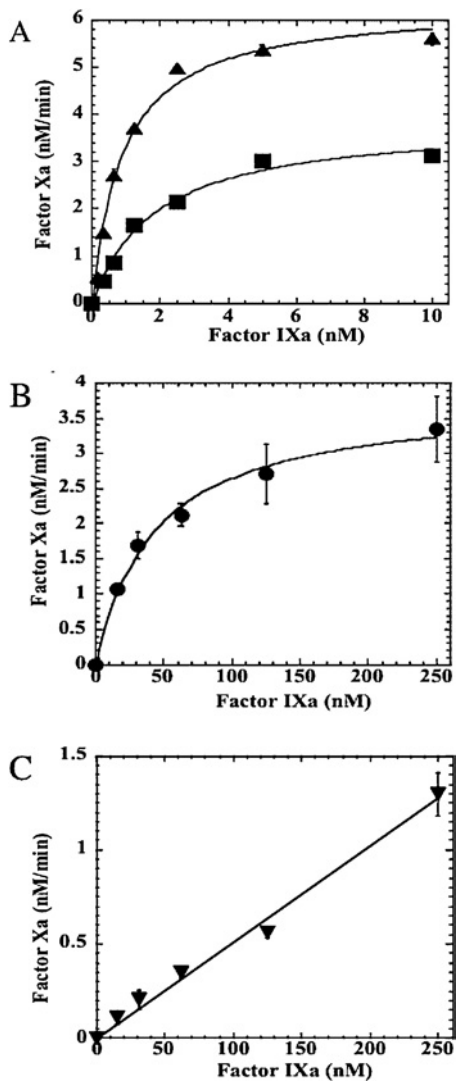


Figure 5 Determination of $K_{d,app(FIXa)}$ and maximal velocity of FX activation (V_{max}^{IXa}) at saturating concentrations of FIXa proteins and on SFLLRN-activated platelets

FIXa proteins were titrated into HT buffer containing SFLLRN-activated platelets (5×10^7/ml). FVIIIa was added to 5 units/ml and the reactions were initiated by addition of FX to 250 nM. After 2 min at 37 °C, the reactions were stopped by addition of EDTA to 10 mM. ▲, $FIXa_N$ (**A**); ■, rFIXa7Ωloop (**A**); ●, rFIXa7EGF2 (**B**); ▼, rFIXa7Ωloop7EGF2 (**C**). Chimeric proteins rFIXa7EGF2 and rFIXa7Ωloop7EGF2 were titrated up to 250 nM, and the results are shown separately in (**B**) and (**C**) for clearer demonstration. The results are expressed as the means ± S.E.M. for three determinations.

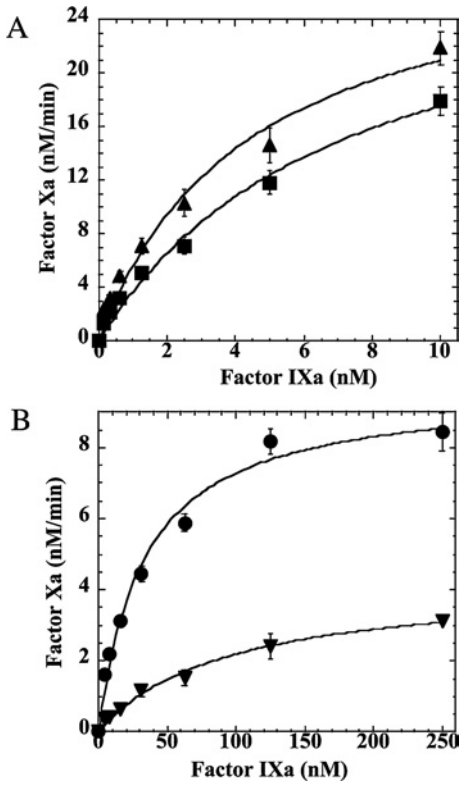


Figure 6 Determination of $K_{d,\ appFIXa}$ and maximal velocity of FX activation (V_{max}^{IXa}) at saturating concentrations of FIXa proteins on phospholipid vesicles (PC/PS, 3:1)

FIXa proteins were titrated into HT buffer containing 1 μM PS/PC. FVIIIa was added to 5 units/ml and the reactions were initiated by addition of FX to 250 nM. After 2 min at 37 °C, the reactions were stopped by addition of EDTA to 10 mM. ▲, $FIXa_N$; ■, rFIXa7Ωloop; ●, rFIXa7EGF2; ▼, rFIXa7Ωloop7EGF2. Chimeric proteins rFIXa7EGF2 and rFIXa7Ωloop7EGF2 were titrated up to 250 nM to achieve saturation or linear regression and the curves are shown separately for clearer demonstration. The results are expressed as the means ± S.E.M. for three determinations.

Table 3 FIXa titrations and kinetic parameters ($K_{d,\ app(FIXa)}$ and V_{max}^{IXa}) for FXa generation by FIXa on SFLLRN-activated platelets and phospholipid vesicles (PC/PS, 3:1)

The results are expressed as the means ± S.E.M. of triplicate measurements for three independent experiments. *$P < 0.05$, †$P < 0.0001$ and ‡P = not significant when compared with $FIXa_N$. ND, not determined.

	Platelets		Phospholipids	
FIXa	$K_{d,\ app(FIXa)}$ (nM)	V_{max}^{IXa} (nM FXa · min $^{-1}$)	$K_{d,\ app(FIXa)}$ (nM)	V_{max}^{IXa} (nM FXa · min $^{-1}$)
$FIXa_N$	0.8 ± 0.01	6.3 ± 0.2	4.4 ± 1.1	30.1 ± 3.5
FIX7Ωloop	1.9 ± 0.3*	3.8 ± 0.2*	7.1 ± 1.6*	30.0 ± 3.6‡
FIX7EGF2	8.2 ± 3.5†	3.5 ± 0.6*	32.5 ± 5.1†	9.7 ± 0.5†
FIX7Ωloop7EGF2	ND	ND	92.5 ± 16.2†	4.3 ± 0.3†

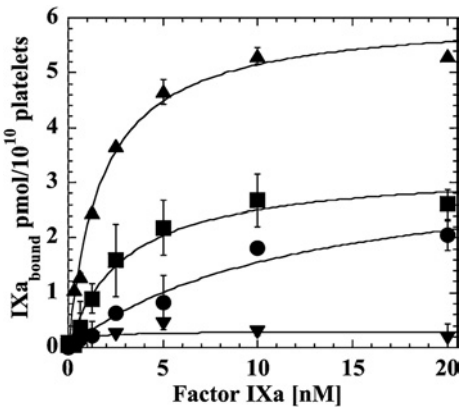


Figure 7 Determination of K_d and B_{max} of FIXa proteins binding to activated platelets

FIXa proteins at indicated concentrations were incubated with SFLLRN-activated platelets (3×10^8/ml), K_d and B_{max} values were determined as described previously [9]. ▲, $FIXa_N$; ■, rFIXa7Ωloop; ●, rFIXa7EGF2; ▼, rFIXa7Ωloop7EGF2. The results are expressed as the means ± S.E.M. for three determinations.

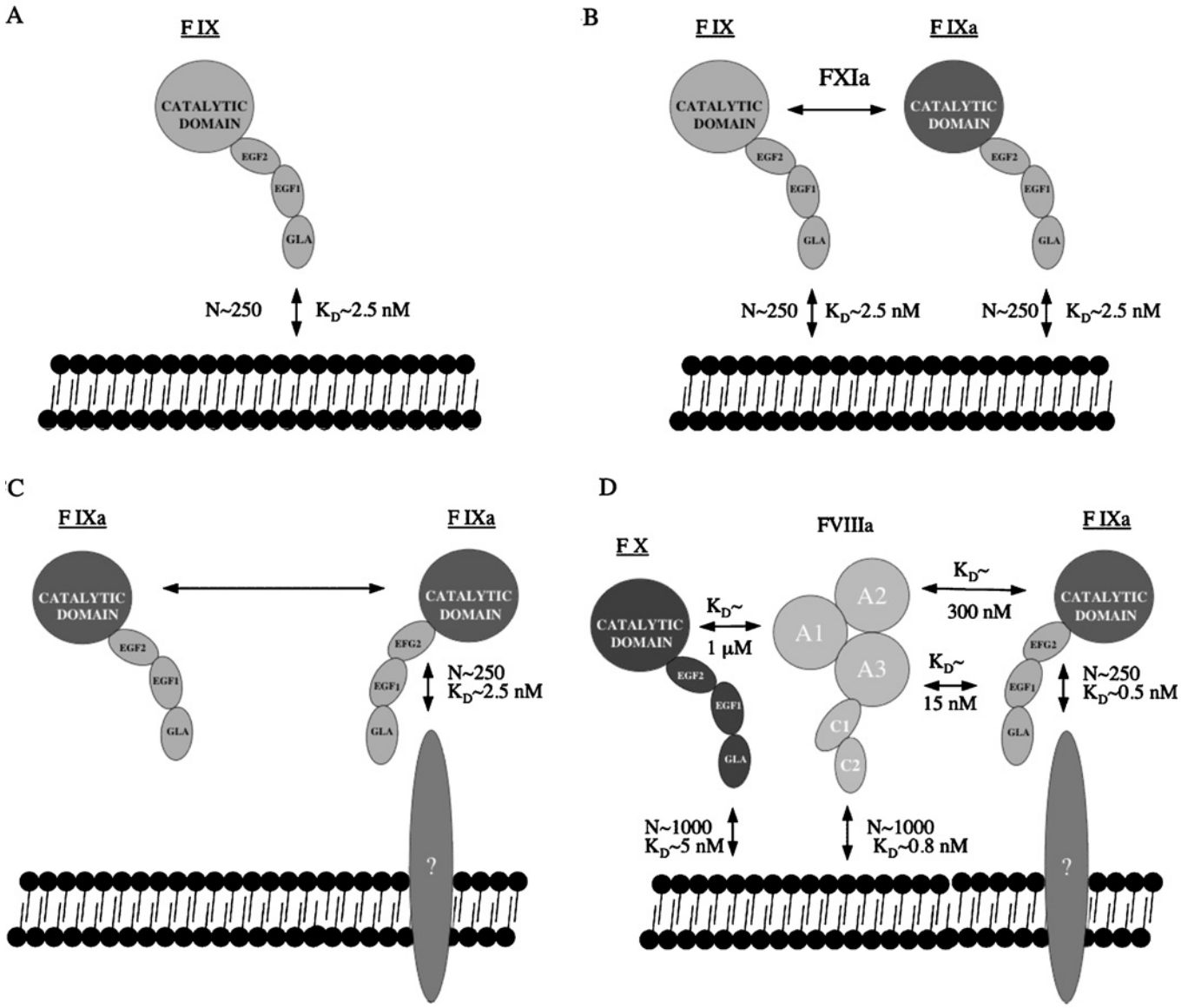
Table 4 Equilibrium binding of FIXa and chimeric FIXa molecules to SFLLRN-activated platelets of FIX/FIXa chimeric proteins

The results are expressed as the means ± S.E.M. of triplicate measurements for three independent experiments. Clotting activities of FIX/FIXa proteins were determined as described in the Experimental section. *$P < 0.01$ and †$P < 0.0001$ when compared with $FIXa_N$. ND, not determined.

FIXa	K_d (nM)	B_{max} (sites/platelet)
$FIXa_N$	1.7 ± 0.2	605 ± 20
FIX7Ωloop	3.6 ± 0.3*	268 ± 78*
FIX7EGF	16.1 ± 4.0†	343 ± 57*
FIX7Ωloop7EGF2	ND	ND

binding to the specific enzyme-binding site, not occupied by the zymogen [9,10,21–29]. Both of these molecular domains have a functional role in the assembly of the FX-activating complex, although the EGF2 domain plays the more direct and predominant part. When FVII residues replaced both sites in FIXa, the resulting protein was completely unable to interact with platelet receptors in the assembly of the FX-activating complex (Figure 3C). It should be noted from the results in Table 2 that when k_{cat} values were calculated as V_{max} (derived from values recorded in Table 2) divided by the concentration of bound enzyme for the FIX7Ωloop and FIX7EGF2 chimeras (derived from values recorded in Table 4), these 'corrected' k_{cat} values were normal. In contrast, a corrected k_{cat} value could not be calculated for the FIX7Ωloop EGF2 chimera, because it did not bind to platelets. These results can be interpreted as demonstrating that the only defect arising from these mutations is a consequence of a platelet-binding defect and that the catalytic activity of the two single chimeras is normal. We conclude that together the Ω-loop of the Gla domain and residues Cys^{88}–Cys^{109} of the EGF2 domain comprise the totality of platelet interactive sites within FIXa.

Scheme 1 shows a postulated sequence of biochemical events leading to the assembly of the FX-activating complex. Initially (Scheme 1A), FIX binds to a discrete number (~250/platelet) of sites with significantly higher affinity ($K_d \sim 2.5$ nM) than its interaction ($K_d \sim 60$ nM) with artificial PS-containing phospholipid vesicles [37,38]. FIX is then activated by FXIa to FIXa, which interacts with the same shared site as that for FIX (Scheme 1B), and is therefore probably rapidly and effectively displaced from this shared site by the relatively higher concentrations of FIX (~90 nM) in plasma. This displacement of the enzyme from the shared site by the zymogen, thereby increasing the local concentration of the enzyme in solution, is likely to account for the interesting observation that zymogen FIX potentiates FIXa-catalysed FX activation by enhancing the apparent



Scheme 1 Model of the assembly of the FX-activating complex

The bilayer represents the phospholipid membrane of platelets; the structures, coagulation proteins are as labelled, with EGF domains, Gla domain and the FVIIIa domains, designated A1, A2, A3, C1, and C2. N, number of binding sites; K_D, dissociation constant. The structure spanning the membrane is undefined with respect to its biochemical nature, as indicated by the question mark. For further explanation, see the text.

affinity of FIXa binding to its platelet receptor [39]. This scenario may also explain the previously confusing observation that a conformationally constrained synthetic peptide (Gly^4–Gln^{11}) mimicking the Ω-loop of the Gla domain is a potent inhibitor ($K_I \sim 2.5$ nM) of FIX/FIXa binding to its shared receptor, but ~50000-fold less effective ($K_I \sim 165$ μM) in inhibiting platelet receptor-mediated FX activation by FIXa [21]. Taken together these facts strongly suggest that FIXa binds to its specific (EGF2-mediated) receptor from solution after dissociation from its shared FIX/FIXa-binding site (Scheme 1C). Thereafter, the affinity of FIXa binding to its EGF2-mediated binding site is enhanced ~5-fold ($K_d \sim 0.5$ nM) in the presence of both FVIIIa and FX, both of which bind to their own specific receptors and interact with one another to assemble the FX-activating complex on the activated platelet surface (Scheme 1D). The intimate functional relationship between these two classes of receptors is underscored by this sequential mechanism of FIX/FIXa interactions with activated platelets. Thus the shared FIX/FIXa-binding site serves not only as a locus for FIX activation by FXIa, but also as a site from which the trace concentrations of FIXa thereby generated can be displaced by the large molar excess of FIX in plasma, thereby increasing the local concentration of FIXa in solution, that is then 'fed' to the specific enzyme-binding site, so that the apparent affinity of FIXa for its specific receptor is further increased [39].

An important issue not directly addressed by the present studies is the biochemical nature of the two classes of interacting binding sites or receptors identified. Simply stated, there are two possibilities to account for each of these two platelet-binding sites and for the high-affinity specific receptors for FVIII [13,14,16]

and FVIIIa [14,16] and for FX [12,14,20], in addition to the shared FX-/prothrombin-binding site [11,12], occupancy of which is also important for promoting optimal rates of FX activation on the activated platelet surface [12]. One of these possibilities, for which there is an enormous amount of supporting data [40–43], is that negatively charged aminophospholipids, not exposed on the external leaflet of the unactivated platelet, are exposed by a complex incompletely understood mechanism involving translocation of lipids in the plasma membranes when platelets are activated by specific agonists or activators [40]. Another possibility to explain some or all of these exposed binding sites is that specific protein receptors are exposed upon platelet activation which contain binding sites that co-localize the components of the FX-activating complex. Although there is no definitive convincing evidence to support the existence of protein binding sites, such receptors could possibly explain the specificity and high-affinity characteristic of each of these classes of ligand-binding sites. Thus for example, FIX and FIXa bind to activated platelets with significantly higher affinity than to phospholipid vesicles [38,44,45], and also with exquisite specificity, whereas the well-documented binding of the Ω-loop of the Gla domain of vitamin-K-dependent coagulation proteins to phospholipid membranes is relatively non-specific in the sense that one protein can displace another [45,46]. Moreover, the binding of FVIII [13,14,16] and FVIIIa [14,16] to activated platelets is highly specific, whereas the homologous protein FV can compete with FVIII for binding to phospholipid vesicles [47]. Nonetheless, in spite of the existence of preliminary evidence of FIX/FIXa-binding proteins exposed in activated platelets [48], none of the published data provides definitive evidence of specific membrane protein receptors for the numerous components of the FX-activating complex. Yet another possibility is the existence of platelet membrane microdomains, such as lipid rafts, exposed upon platelet activation, that contain either lipid or protein components that comprise specific receptors for these components of the FX-activating complex [16,49,50]. In summary it is clear that the various binding sites for FIX, FIXa, FVIII, FVIIIa and FX fulfil the functional requirements for their designation as 'receptors' in the sense that they comprise binding sites that are activation dependent, the exposures of which are closely correlated with profound effects on enzyme kinetic parameters resulting in enormous amplification of blood coagulation. However, no one has so far succeeded in identifying a protein receptor on platelets for any one of these components, which in any case, may not exist.

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REFERENCES

1 Thompson, A. R. (2001) Molecular biology of Factor IX. In Hemostasis and Thrombosis: Basic Principles and Clinical Practice, chapter 7 (Colman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W. and George, J. N., eds.), pp. 123–134, Lippincott Williams & Wilkins, Philadelphia
2 Kaufman, R. J., Antonarakis, S. E. and Fay, P. J. (2001) Factor VIII and hemophilia A. In Hemostasis and Thrombosis: Basic Principles and Clinical Practice, chapter 8 (Colman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W. and George, J. N., eds.), pp. 135–156, Lippincott Williams & Wilkins, Philadelphia
3 Roberts, H. R. and White, Jr, G. C. (2001) Inherted disorders of prothrombin conversion. In Hemostasis and Thrombosis: Basic Principles and Clinical Practice, chapter 52 (Colman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W. and George, D., eds.), pp. 840–853, Lippincott Williams & Wilkins, Philadelphia
4 Rao, A. K. (2001) Acquired qualitative platelet defects. In Hemostasis and Thrombosis: Basic Principles and Clinical Practice, chapter 55 (Colman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W. and George, D., eds.), pp. 905–919, Lippincott Williams & Wilkins, Philadelphia
5 Tracy, P. B. (2001) Role of platelets and leukocytes in coagulation. In Hemostasis and Thrombosis: Basic Principles and Clinical Practice, chapter 33 (Colman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W. and George, J. N., eds.), pp. 575–596, Lippincott Williams & Wilkins, Philadelphia
6 Mertens, K. and Bertina, R. M. (1984) The contribution of Ca^{2+} and phospholipids to the activation of human blood-coagulation Factor X by activated Factor IX. Biochem. J. **223**, 607–615
7 Elodi, S., Varadi, K. and Voros, E. (1981) Kinetics of formation of factor IXa–factor VIII complex on the surface of platelets. Thromb. Res. **21**, 695–700
8 Rawala-Sheikh, R., Ahmad, S. S., Ashby, B. and Walsh, P. N. (1990) Kinetics of coagulation factor X activation by platelet-bound factor IXa. Biochemistry **29**, 2606–2611
9 Ahmad, S. S., Rawala-Sheikh, R. and Walsh, P. N. (1989) Comparative interactions of factor IX and factor IXa with human platelets. J. Biol. Chem. **264**, 3244–3251
10 Ahmad, S. S., Rawala-Sheikh, R. and Walsh, P. N. (1989) Platelet receptor occupancy with factor IXa promotes factor X activation. J. Biol. Chem. **264**, 20012–20016
11 Scandura, J. M., Ahmad, S. S. and Walsh, P. N. (1996) A binding site expressed on the surface of activated human platelets is shared by factor X and prothrombin. Biochemistry **35**, 8890–8902
12 Scandura, J. M. and Walsh, P. N. (1996) Factor X bound to the surface of activated human platelets is preferentially activated by platelet-bound factor IXa. Biochemistry **35**, 8903–8913
13 Nesheim, M. E., Pittman, D. D., Wang, J. H., Slonosky, D., Giles, A. R. and Kaufman, R. J. (1988) The binding of ^{35}S-labeled recombinant factor VIII to activated and unactivated human platelets. J. Biol. Chem. **263**, 16467–16470
14 Ahmad, S. S., Scandura, J. M. and Walsh, P. N. (2000) Structural and functional characterization of platelet receptor-mediated factor VIII binding. J. Biol. Chem. **275**, 13071–13081
15 van Dieijen, G., Tans, G., Rosing, J. and Hemker, H. C. (1981) The role of phospholipid and factor VIIIa in the activation of bovine factor X. J. Biol. Chem. **256**, 3433–3442
16 Ahmad, S. S. and Walsh, P. N. (2002) Lipid raft association of the factor VIII C2 domain mediates platelet membrane assembly of factor VIIIa into the factor-X-activating complex. Blood **100**, 126a
17 Foster, P. A., Fulcher, C. A., Houghten, R. A. and Zimmerman, T. S. (1990) Synthetic factor VIII peptides with amino acid sequences contained within the C2 domain of factor VIII inhibit factor VIII binding to phosphatidylserine. Blood **75**, 1999–2004
18 Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K. and Stoddard, B. L. (1999) Structure of the C2 domain of human factor VIII at 1.5 Å resolution. Nature (London) **402**, 439–442
19 Saenko, E. L. and Scandella, D. (1995) A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor. J. Biol. Chem. **270**, 13826–13833
20 Ahmad, S. S. and Walsh, P. N. (2002) Coordinate binding studies of the substrate (factor X) with the cofactor (factor VIII) in the assembly of the factor X activating complex on the activated platelet surface. Biochemistry **41**, 11269–11276
21 Ahmad, S. S., Wong, M. Y., Rawala, R., Jameson, B. A. and Walsh, P. N. (1998) Coagulation factor IX residues G4–Q11 mediate its interaction with a shared factor IX/IXa binding site on activated platelets but not the assembly of the functional factor X activating complex. Biochemistry **37**, 1671–1679
22 Ahmad, S. S., Rawala, R., Cheung, W. F., Stafford, D. W. and Walsh, P. N. (1995) The role of the second growth-factor domain of human factor IXa in binding to platelets and in factor-X activation. Biochem. J. **310**, 427–431
23 Ahmad, S. S., Rawala-Sheikh, R., Cheung, W. F., Jameson, B. A., Stafford, D. W. and Walsh, P. N. (1994) High-affinity, specific factor IXa binding to platelets is mediated in part by residues 3–11. Biochemistry **33**, 12048–12055
24 Ahmad, S. S., Rawala-Sheikh, R., Monroe, D. M., Roberts, H. R. and Walsh, P. N. (1990) Comparative platelet binding and kinetic studies with normal and variant factor IXa molecules. J. Biol. Chem. **265**, 20907–20911
25 Rawala-Sheikh, R., Ahmad, S. S., Monroe, D. M., Roberts, H. R. and Walsh, P. N. (1992) Role of γ-carboxyglutamic acid residues in the binding of factor IXa to platelets and in factor-X activation. Blood **79**, 398–405
26 Wilkinson, F. H., Ahmad, S. S. and Walsh, P. N. (2002) The factor IXa second epidermal growth factor (EGF2) domain mediates platelet binding and assembly of the factor X activating complex. J. Biol. Chem. **277**, 5734–5741
27 Wilkinson, F. H., London, F. S. and Walsh, P. N. (2002) Residues 88–109 of factor IXa are important for assembly of the factor-X activating complex. J. Biol. Chem. **277**, 5725–5733
28 Wong, M. Y., Gurr, J. A. and Walsh, P. N. (1999) The second epidermal growth factor-like domain of human factor IXa mediates factor IXa binding to platelets and assembly of the factor X activating complex. Biochemistry **38**, 8948–8960

29 Yang, X., Chang, Y.-J., Lin, S.-W. and Walsh, P. N. (2004) Identification of residues Asn^{89}, Ile^{90}, and Val^{107} of the Factor IXa second epidermal growth factor domain that are essential for the assembly of the Factor X-activating complex on activated platelets. J. Biol. Chem. **279**, 46400–46405
30 Geiser, M., Cebe, R., Drewello, D. and Schmitz, R. (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. Biotechniques **31**, 88–92
31 Yan, S. B. (1996) Review of conformation-specific affinity purification methods for plasma vitamin K-dependent proteins. J. Mol. Recognit. **9**, 211–218
32 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76–85
33 Winer, B. J., Brown, D. R. and Michels, K. M. (1991) Statistical Principles in Experimental Design, chapter 7, pp. 158–166, McGraw–Hill, New York
34 Leebeek, F. W., Kappers-Klunne, M. C. and Jie, K. S. (2004) Effective and safe use of recombinant factor VIIa (NovoSeven) in elderly mild haemophilia A patients with high-titre antibodies against factor VIII. Haemophilia **10**, 250–253
35 Hoffman, M. (2003) A cell-based model of coagulation and the role of factor VIIa. Blood Rev. **17** (suppl. 1), S1–S5
36 Monroe, D. M., Hoffman, M., Oliver, J. A. and Roberts, H. R. (1998) A possible mechanism of action of activated factor VII independent of tissue factor. Blood Coagul. Fibrinolysis **9** (suppl. 1), S15–S20
37 London, F. and Walsh, P. N. (1996) The role of electrostatic interactions in the assembly of the factor X activating complex on both activated platelets and negatively-charged phospholipid vesicles. Biochemistry **35**, 12146–12154
38 London, F., Ahmad, S. S. and Walsh, P. N. (1996) Annexin V inhibition of factor IXa-catalyzed factor X activation on human platelets and on negatively-charged phospholipid vesicles. Biochemistry **35**, 16886–16897
39 London, F. S. and Walsh, P. N. (2000) Zymogen factor IX potentiates factor IXa-catalyzed factor X activation. Biochemistry **39**, 9850–9858
40 Zwaal, R. F., Bevers, E. M., Comfurius, P., Rosing, J., Tilly, R. H. and Verhallen, P. F. (1989) Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled red cells: mechanisms and physiological significance. Mol. Cell. Biochem. **91**, 23–31
41 Zwaal, R. F., Comfurius, P. and Bevers, E. M. (1992) Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. Biochim. Biophys. Acta **1180**, 1–8
42 Zwaal, R. F., Comfurius, P. and Bevers, E. M. (1993) Mechanism and function of changes in membrane-phospholipid asymmetry in platelets and erythrocytes. Biochem. Soc. Trans. **21**, 248–253
43 Zwaal, R. F., Comfurius, P. and van Deenen, L. L. (1977) Membrane asymmetry and blood coagulation. Nature (London) **268**, 358–360
44 Christiansen, W. T., Jalbert, L. R., Robertson, R. M., Jhingan, A., Prorok, M. and Castellino, F. J. (1995) Hydrophobic amino acid residues of human anticoagulation protein C that contribute to its functional binding to phospholipid vesicles. Biochemistry **34**, 10376–10382
45 Nelsestuen, G. L., Kisiel, W. and Di Scipio, R. G. (1978) Interaction of vitamin K-dependent proteins with membranes. Biochemistry **17**, 2134–2138
46 Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlback, B. and Nelsestuen, G. L. (1989) Protein structural requirements and properties of membrane binding by γ-carboxyglutamic acid-containing plasma proteins and peptides. J. Biol. Chem. **264**, 20288–20296
47 Gilbert, G. E., Furie, B. C. and Furie, B. (1990) Binding of human factor VIII to phospholipid vesicles. J. Biol. Chem. **265**, 815–822
48 London, F. S. and Walsh, P. N. (1992) Activation-dependent appearance of a platelet protein that recognizes factor IXa. Circulation **86**, I-465
49 Ahmad, S. S. and Walsh, P. N. (2003) Association of factor X with lipid rafts in the presence of active cofactor (factor VIIIa) promotes the assembly of factor-X activating complex on the activated platelet membrane. J. Thromb. Haemost. (suppl. 1), OC114
50 Nguyen, D., London, F. S., Ahmad, S. S. and Walsh, P. N. (2003) Sterol-rich membrane rafts regulate exposure of platelet binding sites for factor IXa. Blood **102**, 772a

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